University of Massachusetts Amherst

From the SelectedWorks of Lynn Margulis (1938 - 2011)

September, 1972

Evidence that the Synchronized Production of New Basal Bodies is not Associated with Dna Synthesis in Stentor Coeruleus

K. B. Younger
S. Banerjee
J. K. Kelleher
M. Winston
Lynn Margulis, *University of Massachusetts - Amherst*

Available at: https://works.bepress.com/lynn_margulis/1/

J. Cell Sci. 11, 621–637 (1972)
Printed in Great Britain

EVIDENCE THAT THE SYNCHRONIZED PRODUCTION OF NEW BASAL BODIES IS NOT ASSOCIATED WITH DNA SYNTHESIS IN *STENTOR COERULEUS*

K. B. YOUNGER, S. BANERJEE, J. K. KELLEHER, M. WINSTON AND LYNN MARGULIS

Department of Biology, Boston University, 2 Cummington Street, Boston 15, Massachusetts 02215, U.S.A.

SUMMARY

Stentors were induced to produce synchronously thousands of new ciliated oral membranellar band basal bodies in less than 3 h. DNA synthesis does not accompany this process, as determined by [^{3}H]thymidine incorporation into isolated bands and by sensitivity to DNA synthesis inhibitors (mitomycin C, ethidium bromide, cytosine arabinoside and hydroxyurea). Yet DNA could be detected in the cortex and the band at basal body sites by autoradiography. Since [^{3}H]thymidine incorporation into membranellar band was eliminated in concentrations of ethidium bromide that had no effect on basal body formation, the previous reports of ciliate kinetosomal (basal body) DNA are interpreted as due to mitochondrial contamination. Specific cortical patterns of DNA that could have been easily misinterpreted as basal body-related were especially apparent in autoradiographs using [^{3}H]actinomycin D as a 'stain'.

In no experiment involving induced basal body regeneration could evidence be found for a correlation between new basal body production and DNA synthesis; RNA and protein synthesis correlated with basal body and cilia regeneration were, however, easily detected by the same techniques. We concluded that there is no evidence that basal body DNA synthesis is required for new basal body production.

INTRODUCTION

The origin and continuity of centrioles and basal bodies (kinetosomes) has been the subject of controversy for many years (Wilson, 1925; Lwoff, 1950; for recent discussions of the evidence see Fulton, 1971; Pickett-Heaps, 1971; Sonneborn, 1970). DNA has been reported at basal body sites in *Tetrahymena* (Randall & Disbrey, 1965); pellicular DNA, autonomously replicating relative to the macronucleus, has been reported in *Paramecium* (Smith-Sonneborn & Plaut, 1967, 1969). The absence of DNA (Hoffman, 1965; Pyne, 1968; Hufnagel, 1969; Flavell & Jones, 1971) in basal body preparations and isolated pellicles has also been reported.

This paper presents different lines of evidence to support the idea that (1) no new DNA synthesis accompanies the formation of 15–20000 new oral membranellar band (MB) basal bodies in regenerating stentors and (2) the DNA previously associated with basal body sites is mitochondrial. An abstract of this work has appeared (Margulis, Banerjee & Younger, 1971).

Our results are more straightforward than those of previous investigators because

of the nature of the oral basal body regeneration system in this heterotrichous ciliate (Tartar, 1957, 1960; Bannerjee & Margulis, 1972). Hundreds of stentors can be induced to shed their oral MBs, structures that contain basal bodies, ciliary axonemes and associated microtubules, and mitochondria (Van Wie, 1969). During 8 h at 24 °C all of the shed cells regenerate the MB in synchrony according to a well known morphogenetic sequence (Tartar, 1960; Paulin & Bussey, 1971). New MB basal bodies are formed between approximately 0·5 and 2·0 h after shedding; the body basal bodies and cilia remain unaltered. Thus metabolism in shed cells, i.e., cells regenerating 15–20000 new basal bodies, can be compared to that in controls, i.e., normal growing cells in which new basal body production has not been induced. Stentors can repeatedly be induced to shed their MBs so that newly regenerated MBs can be collected and examined by direct cytological preparation and autoradiography.

METHODS AND MATERIALS

Cultures and induction of regeneration

Stentor coeruleus, originally kindly supplied by Professor Vance Tartar, have been in culture since 1967. They are maintained on spring water and fed mixed flagellates and ciliates as previously described (Neviackas & Margulis, 1969). They are induced to shed their membranellar bands by addition of 50 % volume of 4 % urea or, when bands are to be collected for cytological study, stentors are shed on slides in sucrose/warm agar medium (see below). Shedding, which occurs in less than 30 s is observed through a Nikon stereozoom microscope. Once it begins to shed the band and its contents continue to lift off: all cells behave alike. Several hundred shedding cells are quickly removed from urea and washed several times through pond water. They are incubated at 24 °C for regeneration which takes from 8–9 h, after which newly regenerated cells are indistinguishable from unshed controls.

Nucleic acid synthesis during regeneration

Stentors were prestarved for several days and healthy organisms as nearly equal in size as possible were withdrawn from the stock cultures for experiment. They were transferred to filtered pond water to which tritiated thymidine had been added (specific activity 6·7–20 Ci/mM) to a final concentration of 10–20 μCi/ml. All radioactive compounds were purchased from New England Nuclear Corporation, Waltham, Massachusetts. Both pre-incubation (before shedding) and post-incubation experiments were undertaken. Incorporation of thymidine into acid-insoluble DNase-sensitive material in both bands and whole cells was measured.

For the RNA experiments cells were preincubated in [^{3}H]uridine or [^{3}H]orotic acid. Several experiments were done in which specific activity of [^{3}H]uridine varied from 3·9 to 6·97 Ci/mM and orotic acid from 9·27 to 12·2 Ci/mM. The final concentration in the stentor medium was 1·0 μCi/ml. They were shed and allowed to regenerate in the presence of the labelled RNA precursor. After 18 h they were shed again and replaced into fresh solution of labelled RNA precursor at the same concentration and after the second regeneration bands were collected for autoradiographic study. A given number of cells (counted by hand) were washed through 5–6 rinses of pond water and reserved for TCA precipitates. These cells were heat killed, solubilized by shaking vigorously in saturated sodium dodecasulphate and 50 % ethanol. They were precipitated in 5 % cold trichloroacetic acid (TCA) on to Millipore (DA 0·65 μm) filters, washed through with TCA (4 °C) and cold water. The dried filters were counted for tritium on a Packard Liquid Scintillation counter. Some heat-killed cells were reserved for incubation in RNase.

Protein synthesis during regeneration

Cells prestarved for 4–5 days were placed into pond water to which food culture and [^{3}H]glutamic acid (specific activity, 1·9 Ci/mM at a final concentration of 1·0 μCi/ml) or [^{3}H]alanine (specific activity 2·93 Ci/mM at a final concentration of 1·0 μCi/ml) had been added. Shedding was induced in the experimentals. The bands were isolated from shed cells after 2 regenerations and from unshed controls. Comparable experiments were done with whole cells: hot-TCA precipitates were made to measure the relative amounts of radioactivity in the hot acid-insoluble fraction of regenerating and non-regenerating cells. Uptake of labelled amino acids into cells lacking the MB is drastically reduced (DeTerra, 1966, and our experience), so for protein studies stentors were pre-incubated in media containing radioactive protein precursors and food before shedding and permitted to regenerate or sit in filtered pond water.

The effects on regeneration of RNA and protein synthesis inhibitors were tested. Washed shed cells at known stages in the regeneration process (Tartar, 1960; Banerjee & Margulis, 1971) were placed into Millipore-filtered pond water to which inhibitors were added. Actinomycin D and cycloheximide were purchased from Sigma Chemical Co., St Louis; daunomycin was a generous gift from Dr I. Wodinsky of the Arthur D. Little Corporation, Cambridge, Mass.

Autoradiographs of isolated ciliated membranellar bands

To study the incorporation of label in nucleic acids or protein in the band, labelled cells were washed through 5–6 rinses of pond water. The washed cells were pipetted on to acid-cleaned slides. An 18 % sucrose solution in 0·5 % agar was heated until the agar suspension dissolved, and as the medium cooled to lukewarm it was gently pipetted on to the live cells. The cells promptly shed their bands on microscope slides. The shed bands were fixed in place by dropwise addition of fixative (1 ml glutaraldehyde, 50 % w/w, in 10 ml phosphate-buffered picric acid-formaldehyde). After 10 min of fixation warm tap water was pipetted on to the slide every 10–15 min for at least 1 h. The slides, left flooded overnight, were dipped in hot water several times to remove as much agar as possible. (This new method of band isolation proved far superior in our hands to the calcium–formol method of DeTerra, 1966.) The pigment granules were then bleached to permit clear observation of autoradiographic grains by dipping the slides in 1 % $KMnO_4$ for 1·5 min. After washing in running water for 1 min the slides were dipped in 5 % oxalic acid for 1 min and rinsed again.

The slides were rinsed through the following solutions 3 times each to remove low-molecular-weight radioactive materials from the bands: 3:1 ethanol–acetic acid, 100 % ethanol and 95 % ethanol. They were air-dried and stored at 4 °C until they were autoradiographed with Kodak AR 10 stripping film. Test exposures of autoradiographed bands were taken, generally after 1 week of exposure. Some slides were reserved for nuclease treatment before autoradiography. Experience showed that ethidium bromide-sensitive thymidine incorporation was removable by acid DNase (Chevremont, Baeckeland & Chevremont-Comhaire, 1960; Chevremont, 1965). DNase buffered at pH 6·8 removed label introduced as thymidine only with very long incubation times and high enzyme concentrations. The following enzyme solutions were used: RNase, grade A crystalline, Calbiochem, 1 mg/ml in McIlvaine's phosphate buffer, pH 6·7–6·9 incubated at 37 °C for 4 h; DNase, bovine pancreatic crystalline DNase I, Calbiochem, 2 mg/ml buffered in McIlvaine's phosphate buffer at pH 6·8–6·9 in 0·002 M $MgSO_4$; or 'acid DNase', spleen DNase II, Miles Laboratories, Kankakee, Illinois, 2 mg/ml buffered at pH 4·6 in acetate buffer in 10^{-4} M $MgSO_4$ and incubated at 37 °C.

[^3H]*Actinomycin D binding*

Approximately 40 stentors were fixed in modified Carnoy (by volume, 6:6:8 ethanol/chloroform/glacial acetic acid) on acid-cleaned slides previously coated with unlabelled actinomycin D, 10 μg/ml (Sigma Chemical Co., St Louis, Mo.), since actinomycin D can stick to glass (Ebstein, 1967). The whole mounted stentors were brought down a graded series of ethanols and washed 3 times in distilled water. Different concentrations from 1·0 to 0·01 mCi/ml ^{3}H-AD ([^{3}H]actinomycin D) in 50 % ethanol–50 % distilled water were tried. ^{3}H-AD was

added dropwise to the whole mounts. The slides were washed through 50 % ethanol, 25 % and 10 %, and several times in distilled water. They were incubated in acid DNase, DNase, RNase, and buffer alone for times up to 24 h. After fixation and enzyme digestion the slides were washed thoroughly, air-dried and stored in the refrigerator until coated with Kodak AR-10 stripping film. For a 1-week exposure of the autoradiogram we found 0·5 mCi/ml at room temperature for about 0·5 h in ^{3}H-AD optimal. All autoradiographs were developed for 10 min in Kodak D-19 at 10–16 °C, rinsed in Kodak stop bath and then in Kodak rapid fix for 2 min.

Inhibitor solutions

Hydroxyurea, mitomycin C, and ethidium bromide were supplied by Calbiochem; cytosine arabinoside was a generous gift from S. Hendler and R. Sanchez of the Salk Institute, La Jolla, California. To assure ourselves that these inhibitors were entering the cell and inhibiting DNA synthesis, growth of stentor cultures in the presence of these drugs was measured. Typically 20 cells were placed into a series of concentrations of the drugs and the growth determined by counts of single cells. When the control cultures under the same environmental conditions had divided approximately twice the experiment was terminated.

Mitochondria staining

To check the effect of ethidium bromide on oral membranellar band mitochondria (at the light-microscopic level) and to confirm the organized presence of mitochondria in the band, isolated bands were mordanted with 3 % potassium dichromate and the pigment granules bleached. These bands on slides were then covered with Altmann's acid-aniline fuchsin (Humason, 1962). The bands were counterstained with methyl green, dehydrated in absolute ethanol, and permanent preparations made.

RESULTS

Cortical DNA

The presence of DNA in the cortex of *S. coeruleus* was clearly shown by both the ^{3}H-AD staining technique and by the incorporation of ^{3}H-T ([^{3}H]thymidine) into DNase-removable material in isolated membranellar bands. The specificity of the ^{3}H-AD label for DNA-containing structures (Plessman-Camargo & Plaut, 1967) was confirmed by entirely black macronuclei in fixed cells covered with 50 μCi/ml ^{3}H-AD solution for less than 1 h and autoradiographed for 4 days. Stripes of cortical label were noted in all of the several hundred whole mounts studied (Figs. 3, 4, 6). These stripes followed the known morphological patterns of stentor kineties (Tartar, 1960). Because cortical pigment granules are in the same size range as the autoradiographic emulsion grains, and the thickness of the preparations varied, no attempt was made to quantitate grain density in whole mount preparations. However in several cases the autoradiographs became slightly displaced and could unequivocally be distinguished from pigment and cell material (Fig. 4). After superimposition of acetate tracings we concluded the pigment stripes were wider than the autoradiographic stripes; the latter were best superimposed at a slight displacement from the pigment stripes. All stentors pretreated with DNase and then stained with ^{3}H-AD showed no macronuclear label and drastically reduced amounts of cortical label relative to control (Figs. 5, 7). Pretreatment with both RNase and DNase resulted in no detectable label above background. Stentors pretreated with RNase alone resembled the controls: they were heavily labelled in both macronucleus and cortex.

The results of the ^{3}H-AD staining of the isolated membranellar bands exactly paralleled the cortical studies: label was distributed in rows corresponding to membranelles and was sensitive to DNase. Invariably labelled material surrounded the isolated MBs, giving the preparations a 'dirty' appearance. This label was due to cell constituents and in some cases organized in patterns reflecting cortical striping (Fig. 14). Background away from the immediate area of the cells was very low; this 'dirty' appearance was not seen in DNase-treated preparations (Fig. 12). Pretreatment with neutrally buffered DNase removed nearly all stainable DNA but only after 24 h in 2 mg/ml enzyme, whereas pretreatment with acid DNase removed all stainable material in 3 h at 37 °C. The MBs and kineties are comprised of organized rows of basal bodies, their cilia, root fibre system and other microtubular derivatives (Bannister & Tatchell, 1968; Paulin & Bussey, 1971). In *Stentor* both the MBs and cortex contain mitochondria aligned in a specific striped array near the basal bodies (Figs. 8, 9). Mitochondria undoubtedly contain double-stranded DNA (see Roodyn & Wilkie, 1968; Ashwell & Work, 1970 for review). This ^{3}H-AD 'staining' which cannot distinguish kinetosomal from cortical mitochondrial DNA demonstrates an organized pattern of cortical and membranellar band DNA quite comparable to the rows of DNase-sensitive grains and fluorescent sites described for *Paramecium* (Randall & Disbrey, 1965) and *Tetrahymena* (Smith-Sonneborn & Plaut, 1967, 1969).

DNA synthesis during regeneration

To distinguish mitochondrial DNA from hypothetical basal body DNA it is necessary to study a system where the production of basal bodies is enriched. This was provided by the comparison between shed-regenerating stentors which produce 15–20000 basal bodies/cell within the first 2 h after induced band regeneration with controls which are only doubling their basal bodies at the same rate as all other cell constituents are doubled. This type of experiment in which ^{3}H-T was incorporated into DNase-sensitive cortical material was done several times in several ways: (1) preincubation of stentors for 1–2 days in medium to which exogenous ^{3}H-T had been added, and (2) incubation of prefed stentors in ^{3}H-T without food after shedding of the MB and removal of the cells 2, 4 and 8 h after induction of basal body regeneration. In several experiments bands isolated from stentors shed twice were compared with those from control stentors that had been incubated for the same total time in ^{3}H-T. Experimentals that had produced approximately 30000 oral basal bodies/cell in 2 days were thus compared with those producing none. Although there was some variation in the total amount of label per isolated band with the various experimental designs the results were the same. In the presence of ^{3}H-T the amount of DNase-sensitive label in the bands of shed cells was, on average, the same as the amount in the controls.

Since the band thymidine label was more sensitive to acid DNase than to neutral DNase and the amount of incorporation was not enriched in bands isolated from newly regenerated cells, we interpreted the cortical label as mitochondrial, not basal body DNA. The 'dirty' pattern of ^{3}H-AD preparations was consistent with the mitochondrial interpretation of cortical DNA (Figs. 13, 14). Ethidium bromide apparently complexes with closed circle DNA (Waring, 1970) and prevents mitochondrial DNA

Table 1. *Comparison of DNA synthesis in shed-regenerating whole cells and unshed controls*

Incubation time in ^{3}H-T before shedding*	TCA precipitate cpm/30 cells	
	Shed twice	Unshed controls
8 h	18	44
	14	12
	9	0
16 h	630	710
	500	780
	540	790

* All cells were prestarved for 72 h and then placed in pond water to which [^{3}H]thymidine (12·5 μCi/ml; specific activity 20 Ci/mM), ethidium bromide (10^{-5} M), and food culture (prelabelled for 3 h in the same ^{3}H-T solution) had been added. After shedding cells were washed 5 times and replaced in unlabelled filtered pond water.

synthesis. Transcription of mitochondrial DNA is prevented in mammalian cells at concentrations that have no effect on nuclear DNA synthesis and transcription (Penman *et al.* 1970; Meyer & Simpson, 1969; Rein & Meyer, 1971). Thus, we began a study of the incorporation of ^{3}H-T into the DNA of isolated MBs in the presence of ethidium bromide.

Shedding of the band was induced and regeneration occurred in several concentrations of ethidium bromide (1×10^{-5}, the borderline of lethality, to 1×10^{-7} M). The fact that basal bodies could be produced normally in concentrations of ethidium bromide that inhibit mitochondrial DNA itself suggested that either there is no basal body DNA or the basal body DNA is sufficiently unlike mitochondrial DNA to be unaffected by ethidium bromide. Incorporation of ^{3}H-T into DNA of isolated MBs could not be clearly detected in the presence of ethidium bromide. This suggests most, if not all, of the MB DNA was a mitochondrial contribution. The preparation of MB stained with acid aniline fuchsin suggested that the number of mitochondria in the bands regenerated in the presence of ethidium bromide was not obviously different from the controls. Presumably redundancy in mitochondrial DNA permits survival and we were observing a reduction in the amount of DNA per mitochondrion without any change in total number of mitochondria. Although cell shape in ethidium bromide was slightly altered, the stentors growing gradually more sickly with time, ethidium bromide (at 10^{-6} M) had no effect on basal body regeneration for at least 4 successively induced regenerations.

Whole-cell experiments indicated that [^{3}H]thymidine is not incorporated into TCA-precipitable material in any greater amount in shed-regenerating cells relative to unshed controls (Table 1). Our results are consistent with Frazier's (1970) observation that starved cells do not synthesize DNA, but synthesis begins in macronuclei about 8 h after feeding. In the presence of ethidium bromide twice-regenerated cells actually incorporated slightly less ^{3}H-T than whole-cell controls. The twice-shed cells have

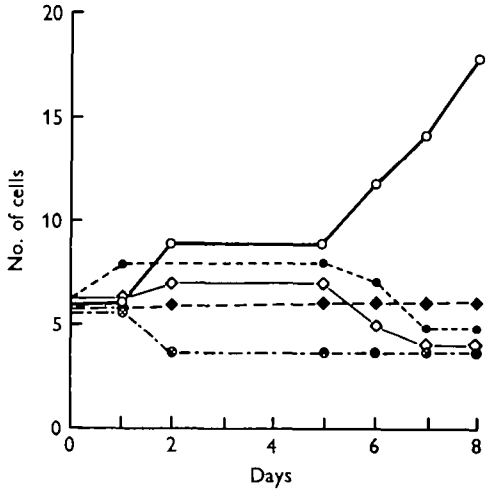


Fig. 1. The growth of well fed stentor in pond water medium containing cytosine arabinoside. Number of cells determined by direct observation. Concentrations of cytosine arabinoside, M; ○—○, zero; ◆--◆, 1×10^{-4}; ●---●, 1×10^{-3}; ◇—◇, 5×10^{-3}; and ⊗-·-·-⊗, 1×10^{-2}.

twice gone through stage 6 (sausage-shaped nucleus that renodulates, Tartar, 1960). Frazier's autoradiographs have shown that DNA synthesis is reduced during this stage. This may account for slightly lower counts in the TCA-precipitable fraction in the shed cell population. In any case if DNA were synthesized during basal body production the predicted result would be exactly opposite to that observed. This is interpreted as further evidence against the hypothesis that basal body production is based on DNA replication.

DNA inhibitors

Shed cells were washed and placed into graded concentrations of DNA synthesis inhibitors. At high concentrations, well above those required to halt growth and division, regenerating cells died. At lower concentrations regeneration was entirely normal. No effect was observed on repeated induced regeneration in the 4 inhibitors. For cytosine arabinoside the growth curves are shown in Fig. 1. Comparable curves were drawn for the other inhibitors. Concentrations which stop growth for a period of time long enough for the control population to double were then tested for their effects on regenerating basal bodies. Hydroxyurea (1, 2, 5×10^{-5} M), mitomycin C (1×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-2} M) and cytosine arabinoside (1×10^{-4}, 5×10^{-3}, 1×10^{-3}, 1×10^{-2} M) all caused eventual cessation of growth, yet had no effect on basal body regeneration. The results are consistent with the interpretation that DNA sensitivity was not observed in cells producing new basal bodies because synthesis of DNA does not accompany the process of basal body production. To be sure that our methods were sensitive enough to detect macromolecular synthesis associated with new oral basal body production, both RNA and protein experiments were undertaken.

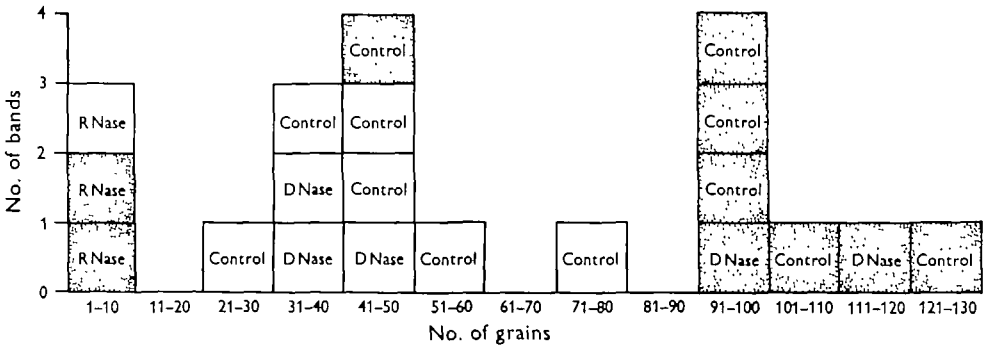


Fig. 2. Frequency distribution of autoradiographic grains counted over bands collected from stentors induced to shed and regenerate twice in the presence of [^{3}H]-orotic acid (specific activity 9·27 Ci/mM; 1 μCi/ml) or unshed. Each box represents one band, stippled boxes represent bands from twice-shed stentors (induced to form new oral band basal bodies) and white boxes represent bands from unshed controls. The number of grains was counted by observation (× 1000) using a monocular grid over a constant area (4 squares in a 22-mm^2 grid divided into 100 squares) of band. Autoradiographs exposed for 7 days.

RNA and protein synthesis during regeneration

With the use of either [^{3}H]uridine or [^{3}H]orotic acid as RNA precursor we could see an enhancement of oral band macromolecular synthesis during regeneration. The frequency distribution of autoradiographic grains in shed and unshed cells is shown in Fig. 2. With either precursor, bands collected from the shed-regenerating cells were qualitatively more labelled than the unshed cells. TCA precipitates of whole cells comparing shed-regenerating cells with unshed controls also indicated RNA synthesis accompanies new basal body production. In both bands and whole cells the shed-regenerating cells were between 1·2 and 2 times as labelled as non-regenerating controls.

Unlike DNA synthesis inhibitors, which have no effect on basal body production, RNA and protein synthesis inhibitors cause a stage-dependent cessation of band regeneration (Table 2). A marked drop in sensitivity to the RNA synthesis inhibitors, daunomycin and actinomycin D, was seen between 1·5 and 2–3 h after shedding, consistent with other work (Burchill, 1968; Frazier, 1970). Cycloheximide sensitivity did not markedly decline until stage 6 (more than 5 h after shedding), confirming the idea that protein synthesis related to band regeneration is not completed until stage 6, whereas RNA synthesis is completed earlier (Burchill, 1968; Frazier, 1970).

Daunomycin apparently acts via the inhibition of RNA synthesis *in vivo* (DiMarco, Silvestrini, DiMarco & Dasdia, 1965). It is likely that this cytotoxic agent (which complexes directly with DNA *in vitro*, Waring, 1970) acts by inhibition of DNA-dependent RNA synthesis, as does actinomycin D. Whether there is specific RNA associated with the basal bodies is not clear from our preparations. The RNA label is generally distributed in the basal regions of the band and could easily be due to contaminating cytoplasmic RNA that comes off with the band.

In an unsuccessful attempt to label ciliary protein with [^{3}H]deoxyguanidine triphosphate (dGTP) or [^{3}H]guanidine triphosphate (GTP) (based on reports of the

Table 2. *Effect of metabolic inhibitors on stentors at various times after induced oral membranellar band regeneration*

Time									
Hours	0	1·0	1·5	2·0	3·0	4·0	5·0		
Stage	PAFF*	1	2		3	4	Early 6	Late 6	7
Inhibitors, molar concentrations									
Actinomycin D									
8×10^{-6}	o	.	o	.	o	o	.	.	.
5×10^{-5}	+	.	+	.	o	o	.	.	.
2×10^{-5}	+	.	+	.	o	o	.	.	.
1×10^{-5}	+	.	+	.	o	o	.	.	.
Daunomycin									
1×10^{-6}	o	o	o	o	.	.	.	.	.
5×10^{-6}	+	+	+	o	o	o	.	.	.
8×10^{-6}	+	+	+	o	o	o	.	.	.
1×10^{-5}	+	+	+	o	o	o	.	.	.
Cycloheximide									
$1{\cdot}8 \times 10^{-5}$	.	+	.	.	.	.	.	.	.
$8{\cdot}9 \times 10^{-5}$	.	.	.	.	.	.	.	d	.
$1{\cdot}8 \times 10^{-4}$	.	+	.	+	+	+	d	d	.
$3{\cdot}6 \times 10^{-4}$†	.	+	.	+	+	+	d	d	o
$7{\cdot}1 \times 10^{-4}$	.	.	.	.	.	.	d	.	o
$1{\cdot}3 \times 10^{-3}$	.	.	.	.	.	.	.	.	o
All DNA synthesis inhibitors	No effect								

**

+, Delay in appearance or migration of band greater than 14 h, abnormal.
o, No effect.
d, 1–3 h delay in migration but normal regeneration.
* Protruding anterior frontal field, immediately after shedding (Tartar, 1960).
† Death within 48 h, whole cells.
** See Burchill (1968) for details.

presence of guanine nucleotide in tubulin, Stephens, 1970) we observed enhanced incorporation of isotope into bands isolated from shed-regenerating cells. This label was RNase sensitive and it was removable entirely with DNase and RNase in combination. We interpreted the dGTP and GTP results as further corroboration that RNA synthesis can be demonstrated to accompany new basal body production.

Protein synthesis detected in whole stentor accompanies MB regeneration (Burchill, 1968); our experiments detected net protein synthesis in isolated bands (Figs. 10, 11). These same results have been seen in hundreds of cells using either [^{3}H]glutamic acid or [^{3}H]alanine as precursor. In spite of cell-to-cell variation, the bands isolated from pre-incubated cells induced to regenerate in the presence of labelled amino acids were more heavily labelled than those in which new band regeneration had not been induced. These experiments convinced us that our results with DNA precursors were genuinely negative under conditions where positive results could be obtained. DeTerra (1966) using [^{3}H]leucine did not observe a comparable clear difference in amount

of label incorporated into bands of shed cells relative to those of unshed controls, perhaps because of the difference in technique of band isolation. Such a dramatic difference between shed-regenerating and control cells with respect to total incorporation of amino acids into protein was difficult to demonstrate in whole-stentor TCA precipitates (Frazier, 1970; Kelleher, 1971), perhaps because the band is a relatively small part of the total cell and protein synthesis occurs maximally in well fed cells whether or not they are forming new band. If the bands are 'dirty' the contribution of labelled adhering cytoplasmic protein is large and a clear-cut difference between the 2 populations apparently can be obscured. However, our results although qualitative, were unequivocal: regardless of precursor, bands from newly regenerated cells were more heavily labelled than those from unshed controls.

DISCUSSION

DNA can be detected in regular patterns on the ciliate cortex and these patterns parallel those of basal bodies. Yet the results of our metabolic experiments suggest that the patterned DNA is mitochondrial, and that 'basal body reproduction' is not dependent on new DNA synthesis. Frazier (1970) has shown that macronuclear DNA synthesis is turned off in starved stentors and others in which the nuclear/cytoplasmic ratio is raised experimentally. Such stentors can regenerate thousands of new oral basal bodies, again implying that DNA synthesis is not associated with basal body production (Frazier, 1970). Although the possibility remains that basal body DNA synthesis is insensitive to hydroxyurea, mitomycin C, ethidium bromide and cytosine arabinoside and that it is below the limits of detectability by light-microscopic autoradiographic techniques, this seems unlikely. Sorokin & Adelstein's (1967) demonstration that 11 J kg^{-1} (1100 rd) of X-radiation failed to affect centriole, basal body and cilia formation in cultured rat lung tissue also suggests that basal body production is not based on DNA replication. It is more likely that the process involving the apparent replication of basal bodies (known to be occurring in the oral MB of stentor after induced regeneration from the elegant scanning and transmission electron-microscopic observations of Paulin & Bussey, 1971) is a 'production' rather than a 'reproduction'. As Sonneborn (1970) notes, not only is DNA synthesis not required to explain the inheritance data but, if detected, it would be difficult to account for. There is no evidence that basal bodies contain a vestigial protein-synthesizing system separate from that of the nucleocytoplasm. Hence Sonneborn's concept that nuclear gene products are precisely patterned on stable inherited cytoplasmic protein templates is the most consistent with our data.

An argument has been presented (Margulis, 1970) for the symbiotic spirochaetal origin of basal bodies and their cilia that were later modified in the evolution of the mitotic apparatus. Obviously the validity of the argument cannot rest on autonomous *in situ* basal body DNA and correlated messenger RNA and protein synthesis, such as is characteristic of mitochondria and chloroplasts. This aspect of the theory of the origin of eukaryotic cells may be incorrect. If correct the original spirochaete genome has been incorporated into the metabolism of the nucleated host: its genes are in the

nucleus and its protein is synthesized off eukaryotic 80-s ribosomes, at least in ciliates. Proof of the theory must come from other sources: homologous spirochaete-eukaryote ribosomal protein, 'motile protein' or ATPase primary sequence data. If total integration of the genes and protein-synthesizing systems of hypothesized host and symbiont is characteristic of advanced eumitotic organisms, a lack of such complete integration may be found in premitotic eukaryotes (Margulis, 1970). If centromeric heterochromatin 'codes for microtubule formation and assembly', a suggestion Craig-Holmes & Shaw (1971) feel deserves consideration, it may be therein that the DNA homologies with the original spirochaete may be found.

We are very grateful for support from NSF grants GB7721 and GB21408 and NASA NGR-004-002. We also thank Sandra Propst for assistance in hydroxyurea experiments, and Professor Fred Schuster (Brooklyn College) for the electron micrographs and helpful discussions.

REFERENCES

ASHWELL, M. & WORK, T. S. (1970). The biogenesis of mitochondria. *A. Rev. Biochem.* **39**, 251–290.

BANERJEE, S. & MARGULIS, L. (1971). Inhibition of cilia regeneration by antineoplastic agents. *Cancer Chemother. Rep.* **55**, 531–537.

BANNISTER, L. H. & TATCHELL, E. C. (1968). Contractility and the fibre system of *Stentor coeruleus*. *J. Cell Sci.* **3**, 295–306.

BURCHILL, B. R. (1968). Synthesis of RNA and protein in relation to oral regeneration in the ciliate *Stentor coeruleus*. *J. exp. Zool.* **169**, 471–480.

CHEVREMONT, M. (1965). Oral comment. In *Use of Radioautography in Investigating Protein Synthesis* (ed. C. P. LeBlond & K. B. Warren), Symp. int. Soc. Cell Biol., vol. 4, p. 105. New York: Academic Press.

CHEVREMONT, M., BAECKELAND, E. & CHEVREMONT-COMHAIRE, S. (1960). Contribution cytochimique et histoautoradiographique a l'étude du métabolisme et de la synthèse des adn dans des cellules animales cultivées *in vitro*. *Biochem. Pharmac.* **4**, 67–78.

CRAIG-HOLMES, A. P. & SHAW, M. W. (1971). Polymorphism of human constitutive heterochromatin. *Science, N.Y.* **174**, 702–704.

DETERRA, N. (1966). Leucine incorporation into the membranellar bands of regenerating and nonregenerating *Stentor*. *Science, N.Y.* **153**, 543–545.

DIMARCO, A., SILVESTRINI, R., DIMARCO, S. & DASDIA, T. (1965). Inhibiting effects of the new cytotoxic antibiotic daunomycin on nucleic acids and mitotic activity of HeLa Cells. *J. Cell Biol.* **27**, 545–550.

EBSTEIN, B. S. (1967). Use of H^3 Actinomycin D as a cytochemical label for small amounts of DNA. *J. Cell Biol.* **35**, 709–713.

FLAVELL, R. A. & JONES, I. G. (1971). DNA from isolated pellicles of *Tetrahymena*. *J. Cell Sci.* **9**, 719–726.

FRAZIER, E. A. J. (1970). *Interactions Between the Nucleus and the Cytoplasm in the Ciliate Stentor coeruleus: An Autoradiographic and Microsurgical Study of Nucleic Acid and Protein Metabolism During Oral Morphogenesis and Macronuclear Replication*. Ph.D. Thesis, University of Washington.

FULTON, C. (1971). Centrioles. In *Origin and Continuity of Cell Organelles* (ed. J. Reinhert & H. Ursprung), pp. 170–221. New York: Springer-Verlag.

HOFFMAN, E. J. (1965). The nucleic acids of basal bodies isolated from *Tetrahymena pyriformis*. *J. Cell Biol.* **25**, 217–228.

HUFNAGEL, L. (1969). Properties of DNA associated with raffinose-isolated pellicles of *Paramecium*. *J. Cell Sci.* **5**, 561–573.

HUMASON, G. L. (1962). *Animal Tissue Techniques*. San Francisco: Freeman & Co.

KELLEHER, J. K. (1971). *Protein Synthesis during Oral Regeneration in Stentor coeruleus*. Master's Thesis, Boston University Graduate School.

LWOFF, A. (1950). *Problems of Morphogenesis in Ciliates*. New York: John Wiley.

MARGULIS, L. (1970). *Origin of Eukaryotic Cells*. New Haven: Yale University Press.

MARGULIS, L., BANERJEE, S. & YOUNGER, K. B. (1971). New basal body production involves RNA and protein synthesis, microtubule formation but not DNA synthesis. *11th A. Meeting Am. Soc. Cell Biol.*, Abstr. 346, p. 177.

MEYER, R. R. & SIMPSON, M. V. (1969). DNA biosynthesis in mitochondria: differential inhibition of mitochondrial and nuclear DNA polymerases by the mutagenic dyes ethidium bromide and acriflavin. *Biochem. biophys. Res. Commun.* **34**, 238–244.

NEVIACKAS, J. A. & MARGULIS, L. (1969). Effect of colchicine on regenerating membranellar cilia in *Stentor*. *J. Protozool.* **16**, 165–171.

PAULIN, J. J. & BUSSEY, J. (1971). Oral regeneration in the ciliate *Stentor coeruleus*: A scanning and transmission electron optical study. *J. Protozool.* **18**, 201–213.

PENMAN, S., FAN, H., PERLMAN, S., ROSBASH, M., WEINBERG, R. & ZYLBER, S. (1970). Distinct RNA synthesis systems of the HeLa cell. *Cold Spring Harb. Symp. quant. Biol.* **35**, 561–577.

PLESSMAN-CAMARGO, E. & PLAUT, W. S. (1967). The radioautographic detection of DNA with tritiated actinomycin D. *J. Cell Biol.* **35**, 713–714.

PICKETT-HEAPS, J. (1971). Autonomy of the centriole: fact or fallacy? *Cytobios* **3**, 205–214.

PYNE, M. C. (1968). Sur l'absence d'incorporation de la thymidine tritiée dans les cinétosomes de *Tetrahymena pyriformis* (Ciliés Holotriches). *C. r. hebd. Séanc. Acad. Sci., Paris* **267**, 755–757.

RANDALL, J. T. & DISBREY, C. (1965). Evidence for the presence of DNA at basal body sites in *Tetrahymena pyriformis*. *Proc. R. Soc.* B **162**, 473–491.

REIN, D. C. & MEYER, R. R. (1971). Ethidium bromide and the biogenesis of mitochondria in *Tetrahymena*. *11th A. Meeting Am. Soc. Cell Biol.*, Abstr. 478, p. 243.

RODYN, D. B. & WILKIE, D. (1968). *The Biogenesis of Mitochondria*. London: Methuen.

SMITH-SONNEBORN, J. & PLAUT, W. (1967). Evidence for the presence of DNA in the pellicle of *Paramecium*. *J. Cell Sci.* **2**, 225–234.

SMITH-SONNEBORN, J. & PLAUT, W. (1969). Studies on the autonomy of pellicular DNA in *Paramecium*. *J. Cell Sci.* **5**, 365–372.

SONNEBORN, T. M. (1970). Determination, development and inheritance of the structure of the cell cortex. *Symp. Soc. dev. Biol.* **9**, 1–13. New York: Academic Press.

SOROKIN, S. P. & ADELSTEIN, S. J. (1967). Failure of 1100 Rads of X-Radiation to affect ciliogenesis and centriolar formation in cultured rat lungs. *Radiat. Res.* **31**, 748–759.

STEPHENS, R. E. (1970). Microtubules. In *Biological Macromolecules*, vol. 5 (ed. S. N. Timasheff & G. D. Fasman), pp. 355–391. New York: Dekker.

TARTAR, V. (1957). Reactions of *Stentor coeruleus* to certain substances added to the medium. *Expl Cell Res.* **13**, 317–322.

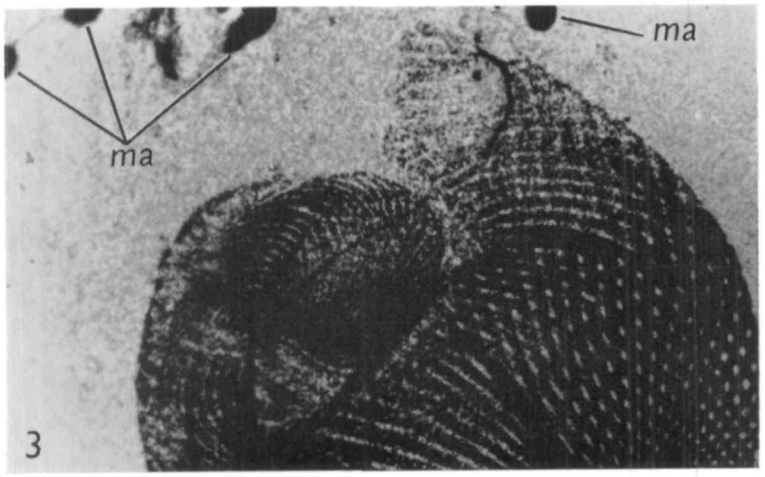
TARTAR, V. (1960). *The Biology of Stentor*. New York: Pergamon Press.

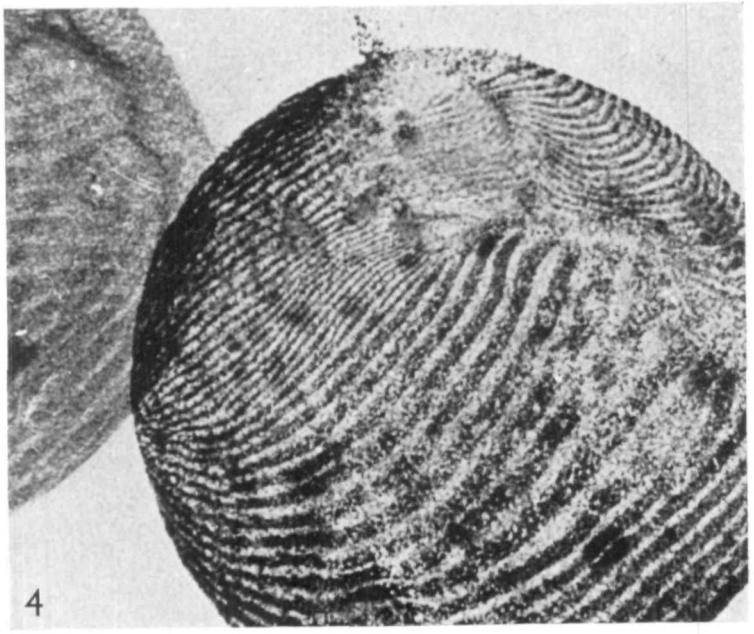
VAN WIE, C. (1969). *An Electron Microscopic Investigation of Intact and Shed Membranellar Bands of Stentor coeruleus*. Master's Thesis, Boston University Graduate School.

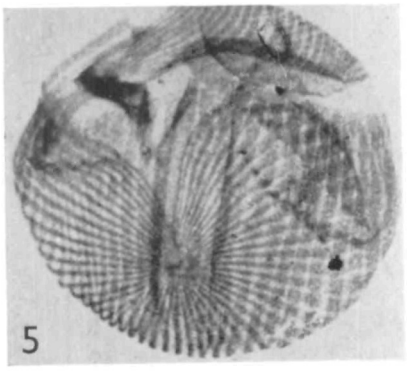
WARING, M. J. (1970). Antimicrobial drugs which inhibit DNA synthesis. In *Macromolecules: Biosynthesis and Function* (ed. S. Ochoa, C. Asensio, C. F. Heredin & D. Nachmansohn), Fedn Eur. Biochem. Soc. Symp., vol. 21, pp. 143–153. New York: Academic Press.

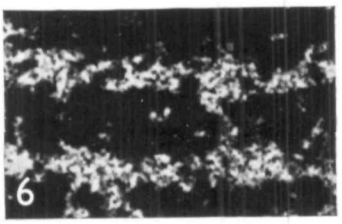
WILSON, E. B. (1925). *The Cell in Development and Heredity*. New York: Macmillan.

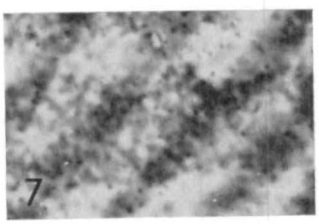
(*Received* 3 *January* 1972)











Figs. 3–7. For legend see p. 634.

Fig. 3. Autoradiogram of control stentor stained with ^{3}H-AD, showing pattern of cortical label and density of macronuclear label. During fixation the intact, beaded macronucleus was squashed out of the cell. The intense label in 4 macronuclear nodes (*ma*), and the connexion between them can be seen above cell. × 100.

Fig. 4. Whole-mount stentor (left) fixed in modified Carnoy and stained for 1 h with ^{3}H-AD, 1 mCi/ml. Autoradiogram (right) of same cell which has been slightly displaced. 1 week exposure of autoradiogram. This type of patterned cortical label was observed in all control cells. × 100.

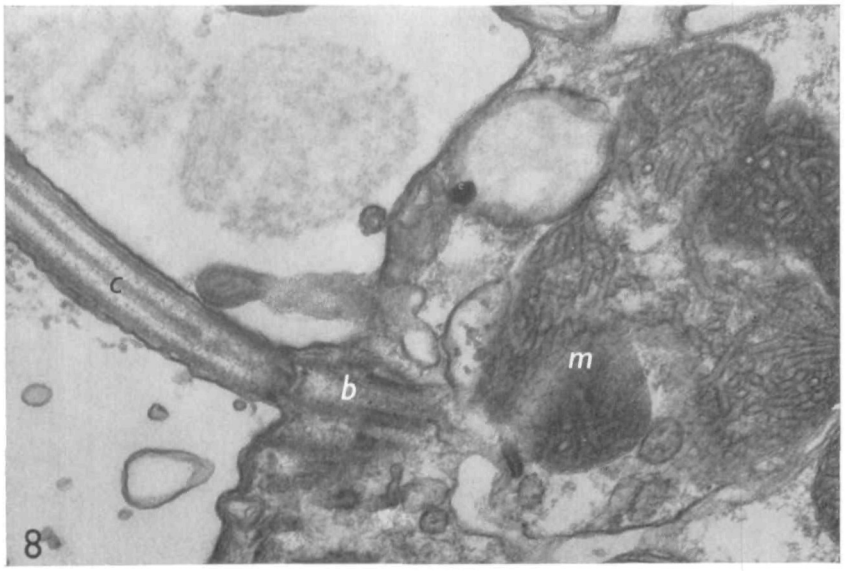
Fig. 5. Typical autoradiogram of whole-mount stentor fixed in modified Carnoy, pretreated with 2 mg/ml DNase for 17 h, and stained with ^{3}H-AD. 3 week exposure of autoradiogram. Macronuclei are unlabelled. × 100.

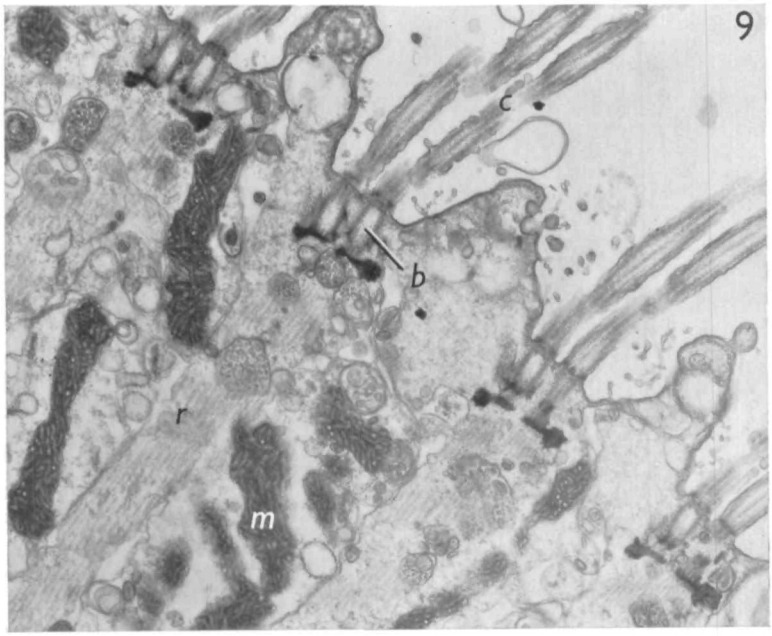
Fig. 6. Cortical label in stentor stained with ^{3}H-AD for comparison with Fig. 7. × 1000.

Fig. 7. Autoradiogram of cortex of DNase-pretreated stentor stained with ^{3}H-AD as in Fig. 5. The amount of cortical label is drastically reduced relative to the controls in Figs. 3, 4, 6. × 1000.

Fig. 8. Ultrastructure of *Stentor coeruleus* cortex showing relationship of mitochondria to basal bodies of body cilia. (× 35000, courtesy Prof. F. L. Schuster.) *b*, basal body; *c*, body cilium; *m*, mitochondrion.

Fig. 9. Ultrastructure of *Stentor coeruleus* cortex showing relationship of mitochondria to paired cilia of the membranellar band. (× 13000, courtesy Prof. F. L. Schuster.) *b*, basal body; *c*, oral cilium; *m*, mitochondrion; *r*, root fibre system of the membranelle.


c
b
m
8


9
c
b
r
m

Figs. 10, 11. Autoradiographs of isolated oral membranellar bands (*mb*) from prefed stentors incubated in the presence of tritiated amino acids. *c*, oral band cilia. × 1000.

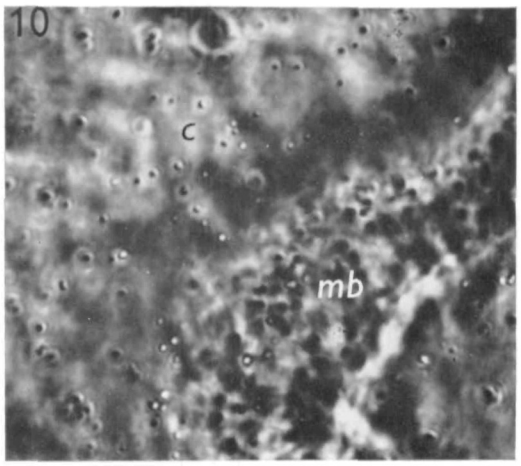
Fig. 10. Band from unshed cell preincubated for 2 days in [^{3}H]glutamic acid.

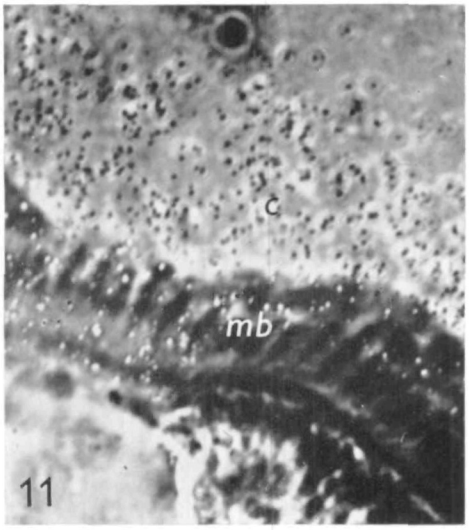
Fig. 11. Band from cell shed and regenerated twice, preincubated for 2 days in the presence of [^{3}H]alanine.

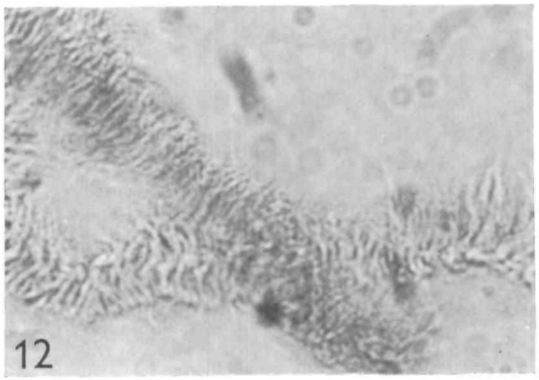
Fig. 12. Same as Fig. 13 but the isolated membranellar band was preincubated in DNase for 17 h. × 1000.

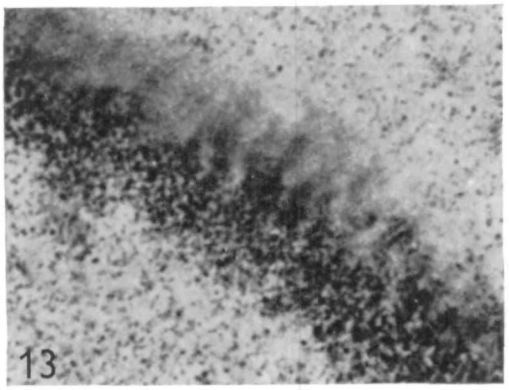
Fig. 13. Autoradiograph of isolated membranellar band stained with ^{3}H-AD. Over 50 isolated bands showed this same pattern of stained membranelles and high background. × 1000.

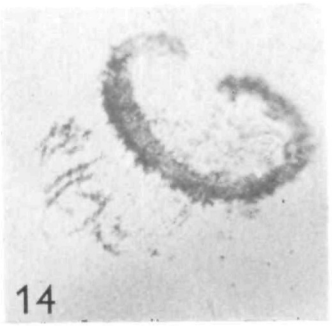
Fig. 14. Autoradiograph only of an isolated membranellar band and some adhering cortex. The cell was stained with ^{3}H-AD (0·5 h, 1 mCi/ml, 3-day exposure of the film at 4 °C). The typical high background may be due to mitochondrial DNA attached to membrane fragments. × 100.


10
c
mb


c
mb
11


12


13


14